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METHOD AND PRODUCT FOR THE SEQUENCE DETERMINATION
OF PEPTIDES USING A MASS SPECTROMETER

RELATED APPLICATION

5   This application is a continuation in part of
copending and commonly owned application serial number
07/891,177 filed May 29, 1992.

FIELD OF THE INVENTION

         This invention relates to rapid and efficient
10   methods for sequencing formed or forming polypeptides
utilizing a mass spectrometer.

         Polypeptides are a class of compounds composed of
α-amino acid residues chemically bonded together by amide
linkages with elimination of water between the carboxy
15   group of one amino acid and the amino group of another
amino acid. A polypeptide is thus a polymer of α-amino
acid residues which may contain a large number of such
residues. Peptides are similar to polypeptides, except
that they are comprised of a lesser number of α-amino
20   acids. There is no clear-cut distinction between
polypeptides and peptides. For convenience, in this
disclosure and claims, the term "polypeptide" will be
used to refer generally to peptides and polypeptides.

Proteins are polypeptide chains folded into a defined three dimensional structure. They are complex high polymers containing carbon, hydrogen, nitrogen, and sulfur and are comprised of linear chains of amino acids connected by peptide links. They are similar to polypeptides, but of a much higher molecular weight.

For a complete understanding of physiological reactions involving proteins it is often necessary to understand their structure. There are a number of facets to the structure of proteins. These are the primary structure which is concerned with amino acid sequence in the protein chain and the secondary, tertiary and quaternary structures which generally relate to the three dimensional configuration of proteins. This invention is concerned with sequencing polypeptides to assist in determining the primary structure of proteins. It provides a facile and accurate procedure for sequencing polypeptides. It is also applicable to sequencing the amino acid residues at the termini of proteins.

Many procedures have been used over the years to determine the amino acid sequence, i.e. the primary structure, of polypeptides and proteins. At the present time, the best method available for such determinations

is the Edman degradation. In this procedure, one amino
terminal amino acid residue at a time is removed from a
polypeptide to be analyzed. That amino acid is normally
identified by reverse phase high performance liquid

5   chromatography (HPLC), but recently mass spectrometric
procedures have been described for this purpose (1).
The Edman degradation cycle is repeated for each
successive terminal amino acid residue until the
complete polypeptide has been degraded. The procedure

10   is tedious and time consuming. Each sequential removal
of a terminal amino acid requires 20 to 30 minutes.
Hence, with a polypeptide of even moderate length, say
for example 50 amino acid residues, a sequence
determination may require many hours. The procedure has

15   been automated. The automated machines are available as
sequenators, but it still requires an unacceptable
amount of time to carry out a sequence analysis.
Although the procedure is widely employed, one which
required less time and which yielded information about a

20   broader range of modified or unusual amino acid residues
present in a polypeptide would be very useful to the
art. A process which can be used to sequence individual
members of mixtures of polypeptides would be
particularly useful.

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Recent advances in the art of mass spectroscopy
have made it possible to obtain characterizing data from
extremely small amounts of polypeptide samples. It is,
for example, presently possible because of the

5   sensitivity and precision of available instruments to
obtain useful data utilizing from picomole to
subpicomole amounts of products to be analyzed.
Further, the incipient ion-trap technologies promise
even better sensitivities, and have already been

10   demonstrated to yield useful spectra in the 10^{-15} to
10^{-16} sample range.

In general, both electrospray and matrix-assisted
laser desorption ionizaton methods mainly generate
intact molecular ions. The resolution of the

15   electrospray quadrupole instruments is about 1 in 2,000
and that of the laser desorption time-of-flight
instruments about 1 in 400. Both techniques give mass
accuracies of about 1 in 10-20,000 (i.e. +/- 0.01% or
better). There are proposed modifications of time-of-

20   flight analyzer that may improve the resolution by up to
factor of 10-fold, and markedly improve the sensitivity
of that technique.

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These techniques yield mass measurements accurate
to +/- 0.2 atomic mass units, or better. These
capabilities mean that, by employing the process of this
invention, the polypeptide itself whether already formed

5   or as it is being formed can be sequenced more readily,
with greater speed, sensitivity, and precision, than the
amino acid derivative released by stepwise degradation
techniques such as the Edman degradation. As will be
explained in more detail below, the process of this

10   invention employs a novel technique of sequence
determination in which a mixture containing a family of
"fragments", each differing by a single amino acid
residue is produced and thereafter analyzed by mass
spectroscopy.

15   SUMMARY OF THE INVENTION

This invention provides a method for the sequential
analysis of polypeptides which may be already formed or
are being formed by producing under controlled
conditions, from the formed polypeptide or from the

20   segments of the polypeptide as it is being formed, a
mixture containing a series of adjacent polypeptides in
which each member of the series differs from the next
adjacent member by one amino acid residue. The mixture
is then subjected to mass spectrometric analysis to

25   generate a spectrum in which the peaks represent the

separate members of the series. The differences in
molecular mass between such adjacent members coupled
with the position of the peaks in the spectrum for such
adjacent members is indicative of the identity of the

5   said amino acid residue and of its position in the chain
of the formed or forming polypeptide.

The process of this invention which utilizes
controlled cycling of reaction conditions to produce
peptide ladders of predictable structure is to be

10   contrasted with previous methods employing mass
spectroscopy including exopeptidase digestion on
uncontrolled chemical degradation. See references 2-5.
Because of the uncontrolled nature of these previous
methods, only incomplete sequence information could be

15   obtained.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 indicates a family or mixture of
polypeptides (peptide ladder, as defined hereinafter)
derived from a single formed polypeptide containing n

20   amino acid residues. The mixture is analyzed in
accordance with this invention to determine the amino
acid sequence of the original polypeptide. Each amino

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acid in the sequence is denoted by a number with the
numbering starting at the amino terminal of the peptide.
X denotes a terminating group.

5   Fig. 2 is an idealized mass spectrum of the peptide
ladder of a polypeptide similar to the family shown in
Fig. 1.

       Fig. 3 shows the reactions involved in generating a
peptide ladder from a formed polypeptide for analysis
utilizing phenyl isothiocyanate (PITC) as the coupling
10   reagent and phenyl isocyanate (PIC) as the terminating
reagent.

       Fig. 4 is a more precise summary of the process
shown in Fig. 3.

       Fig. 5 is an idealized mass spectrum of peptide
15   ladders obtained from a mixture of two formed
polypeptides one of which is identified as A, the other
as B.

       Fig. 6 is a positive ion, matrix assisted laser
desorption mass spectrum of the formed polypeptide
20   [Glu^1]fibrinopeptide B.

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Fig. 7 is a positive ion matrix assisted laser
desorption spectrum of [Glu^1]fibrinopeptide B after 7
cycles of sequential reactions in accordance with an
embodiment this invention in which a formed polypeptide
5   is degraded in a controled manner to produce a mixture
containing a peptide ladder.

Fig. 8 is the spectrum of the peptide ladder in the
region 87-67 obtained from the mixture 99-67 in Example
2.

10   Fig. 9 is the spectrum of the mixture 66-33
obtained in Example 2.

Fig. 10 is a spectrum of the low mass region
obtained from the mixture 66-33 obtained in Example 2
showing the side reaction products formed during the
15   synthesis of HIV-1 protease.

Fig. 11 is a spectrum of the reaction mixture
obtained in Example 3.

Figs. 12A and 12B show the reaction support system
employed in an embodiment of the inventions which
20   permits multiple simultaneous sequencing of
polypeptides.

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Figs. 13A and 13B are the mass spectra of the peptide ladders formed from both phosphorylated (12A) and unphosphorylated (12B) 16 residue peptides containing a serine residue.

5   Fig. 14 shows the spectrum of a protein ladder generated by incomplete Edman degradation.

Fig. 15 shows the spectrum of the mixture obtained in Example 4.

As will be explained in more detail below, Figs. 8
10   through 10 are spectra obtained in the sequencing of a forming polypeptide employing the process of this invention.

The invention will be more easily understood if certain of the terms used in this specification and
15   claims are defined.

The term "polypeptide" is used herein in a generic sense to describe both high and low molecular weight products comprising linear covalent polymers of amino acid residues. As the description of this invention
20   proceeds, it will be seen that mixtures are produced which may contain individual components containing 100

or more amino acid residues or as few as one or two such
residues. Conventionally, such low molecular weight
products would be referred to a amino acids, dipeptides,
tripeptides, etc. However, for convenience herein, all

5   such products will be referred to as polypeptides since
the mixtures which are prepared for mass spectrometric
analysis contain such components together with products
of sufficiently high molecular weight to be
conventionally identified as polypeptides.

10   The term "formed polypeptide" refers to an existing
polypeptide which is to be sequenced. It refers, for
example to [Glu^1]fibrinopeptide B which is sequenced for
purposes of illustration in Example 1. The process of
the invention is, of course, most useful for sequencing

15   the primary structure of unknown polypeptides isolated,
for example, by reverse phase HPLC of an enzymatic
digest from a protein.

     The term "forming polypeptide" refers to such
polypeptides as they are being formed for example by

20   solid phase synthesis as illustrated in Example 2.

     The term "peptide ladder" refers to a mixture
containing a series of polypeptides produced by the
processes described herein either from a formed or a

forming polypeptide. As will be seen from the various

figures and understood from this description of the

invention, a peptide ladder comprises a mixture of

polypeptides in which the various components of the

5   mixture differ from the next adjacent member of the

series by the molecular mass of one amino acid residue.

        A "coupling reagent" is a reactant which forms a

reaction product with a terminal amino acid residue of a

polypeptide to be sequenced and is subsequently removed

10   together with the residue.

        A "terminating reagent" is a reactant which

similarly forms a reaction product with a terminal amino

acid of polypeptide and is stable to subsequent cycling

procedures.

15   DETAILED DESCRIPTION OF THE INVENTION

        There are several procedures for building peptide

ladders, some applicable to the sequencing of formed

polypeptides, others to sequencing of polypeptides as

they are being formed.

20   One such process will be understood from a study of

Fig. 3 which shows an embodiment of the invention which

is applicable to formed polypeptides. The figure shows

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the sequencing of an original formed polypeptide which
may contain any number of amino acid residues, even as
many as 50 or more. The polypeptide is shown here by
way of illustration as containing three residues, each

5   residue with a side chain represented by R_1, R_2 or R_3 in
accordance with conventional practice.

The significant feature of this embodiment of the
invention, as illustrated in the figure, is that the
reaction conditions are cycled to produce a peptide

10   ladder in the final mixture. The final mixture is
analyzed by mass spectroscopy to determine the exact
mass of the components of the ladder, thereby to
accumulate the information necessary to sequence the
original polypeptide.

15   The skilled artisan will recognize that this
procedure of sequencing a formed polypeptide makes use
of degradation chemistry, but is based on a new
principle, i.e. the original polypeptide is employed to
generate a family of fragments, each differing by a

20   single amino acid as shown in Fig. 1 wherein X
represents a terminating agent. Typically X will be a
terminating agent that is resistant to all subsequent
reactions or manipulations in the cyclic degradation

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process of this invention. As will be described below,
in connection with another embodiment of this invention,
X may also be hydrogen.

5   In the process illustrated in Fig. 3, PITC is the
coupling reagent and PIC is the terminating reagent.
From such a family or peptide ladder of terminated
molecular species prepared as outlined in the figure,
the amino acid sequence can be simply read out in a
single mass spectrometry operation, based on the mass
10   differences between the intact molecular ions.
Furthermore, because of the sensitivity of modern mass
spectrometers, the accuracy of the amino acid sequence
thus determined is unaffected, over a wide range (5-fold
or more), by the amount of each molecular species
15   present in the mixture.

Fig. 2 shows an idealized mass spectrum of a
peptide ladder in which each peak is representative of
one member of a series of terminated polypeptides each
member of which differs from the adjacent member by one
20   amino acid residue.

Thus, for example, if the peaks of the highest mass
in Fig. 2 represent a polypeptide, the first five
members of which at the amino terminal end may be:

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$$Gly^1-Leu-Val-Phe-Ala^5-,$$

the next peak of lower mass would represent

$$Leu^2-Val-Phe-Ala^5-$$

Subsequent peaks would represent products with one less

5   amino acid residue. The difference in mass between

adjacent members of the series would be indicative of

the amino acid residue removed. The difference in

molecular mass between the first product on the right

and the adjacent product would correspond to a glycine

10   residue. Subsequent peaks show the sequential removal

of leucine, valine, phenylalanine and alanine residues

thus establishing the sequence of these amino acid

residues in the original polypeptide.

Fig. 3 illustrates a practical sequence of

15   reactions by which the idealized procedure of Figs. 1

and 2 can be conducted utilizing PITC and PIC as the

reagents for sequencing an original formed polypeptide

by cycling reaction conditions to produce a peptide

ladder for spectrometric analysis.

20   In the first step of the sequencing procedure the

original polypeptide is reacted with a mixture of PITC

and PIC under basic conditions. A large molar excess of

each reagent is employed. A much larger amount of PITC

than of PIC is utilized so as to be certain that at each

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cycle of the procedure most of the available polypeptide

reacts with the coupling agent but that a small

measurable fraction of the available peptide reacts with

the terminating reagent. The fraction reacted with the

5   terminating agent will be determined by the relative

activities of the coupling agent and the terminating

agent, and the molar ratio of the two reagents.

The first reaction products which form during the

basic step of the cycle comprise a mixture of original

10   polypeptide terminated with PIC (PC-polypeptide) and an

original polypeptide terminated with PITC (PTC-

polypeptide). The PIC terminated polypeptide (PC-

polypeptide) is stable or essentially stable under all

subsequent reaction conditions with the result that it

15   will be present in a measureable amount in the final

mixture when that mixture is ready for analysis.

The next step in the procedure is to subject the

PTC-polypeptide/PC-polypeptide mixture to acid

conditions whereupon a reaction product separates from

20   the PTC-polypeptide. This reaction product contains the

terminal amino acid residue of the original peptide.

The separation of this product results in the formation

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of a new polypeptide which, because the terminal amino

acid has been cleaved contains one less amino acid than

the original polypeptide.

The reaction mixture formed at the end of this

5   cycle contains as the principal products:

1.   unreacted coupling and terminating

reagents,

2.   a first reaction product which is the

reaction product between the original

10   polypeptide and the terminating reagent.   It

is a PC terminated polypeptide (PC-

polypeptide).

3.   a new polypeptide from which the amino

terminal amino acid residue has been removed.

15   The skilled artisan will readily understand that

sequential repeats of the cycle just described will

result in the formation of a mixture which contains as

the principal measureable components a series of PC-

polypeptides each member of which contains one less

20   amino acid residue than the next higher member of the

series.   The member of the series with the highest

molecular mass will be the first reaction product

between the original polypeptide and the terminating

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reagent. The molecular mass of each subsequent reaction product in the series will be the molecular mass of the next higher adjacent member of the series minus the molecular mass of the terminal amino acid residue

5   removed by reaction with the PITC. The molecular mass of the PIC, blocking group or any other blocking group selected is irrelevant to the spectrometric analysis since the identity of each amino acid residue removed from the next adjacent peptide is determined by

10 differences in molecular mass. These differences identify the amino acid residue, and the position of that mass difference in the spectrum data set defines the position of the identified residue in the original polypeptide.

15   A constant 5% termination of the available polypeptide at each cycle for ten cycles of the described chemistry would yield a peptide ladder in which the mole fraction of the original polypeptide after each cycle would be approximately

	MOLE FRACTION
(X)-1-2-3-4-5-6-7-8-9-10-11-12-.........-n-(OH)	.050
(X)-2-3-4-5-6-7-8-9-10-11-12-..........-n-(OH)	.048
(X)-3-4-5-6-7-8-9-10-11-12-..........-n-(OH)	.045
(X)-4-5-6-7-8-9-10-11-12-..........-n-(OH)	.043
(X)-5-6-7-8-9-10-11-12-..........-n-(OH)	.041
(X)-6-7-8-9-10-11-12-..........-n-(OH)	.039
(X)-7-8-9-10-11-12-...........-n-(OH)	.037
(X)-8-9-10-11-12-..........-n-(OH)	.035

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(X)-9-10-11-12-..........-n-(OH)        .033
   (X)-10-11-12-..........-n-(OH)        .031
      (X)-11-12-............-n-(OH)        .60
                                      remains
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5   The differences in molecular mass between each

successive member of the series in the peptide ladder

can be readily determined with high precision by mass

spectroscopy.

         With relatively low molecular weight polypeptides,

10   it is possible to repeat each cycle without removal of

unreacted PITC or PIC. However, as illustrated in

Example 1, it is generally preferred to remove unreacted

coupling and terminating reagents at the completion of

each cycle. Such removal may also include removal of

15   the cleavage reaction product between the coupling

reagent and the terminal amino acid.

         Fig. 4 is a more precise summary of the procedure

illustrated in Fig. 3 and described in detail above. It

specifically illustrates the process utilizing a "one

20   pot" technique. In the figure "AA" stands for amino

acid and ATZ represents 5-anilinothiazolinone. The

other symbols have the same meaning as above.

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The figure illustrates the preparation of a peptide

ladder from a formed polypeptide using controlled

ladder-generating chemistry. The stepwise degradation

is conducted with a small amount of PIC and a major

5   proportion of PITC. Successive cycles of peptide ladder

generating chemistry are performed as described above

without intermediate isolation or analysis of released

amino acid derivatives. Finally the mixture containing

the peptide ladder is read out in one step by laser

10   desorption time-of-flight mass spectrometry (LDMS).

The coupling and terminating reagents are not

limited to the pair described above. Those skilled in

the art can readily select other equivalent reagents.

Of course, the procedure can be adapted to either the

15   amino terminal or the carboxy terminal of the

polypeptide under analysis.

Another procedure for constructing a peptide ladder

from a formed polypeptide is to conduct each cycle in a

manner to insure incomplete termination. The process is

20   similar to the above described procedure except that

only a coupling reagent is employed and the peptide

ladder comprises a series of polypeptides none of which

is terminated with a terminating reagent but each of

which differs from the adjacent member of the series by

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one amino acid residue. In this procedure, X of Fig. 1
is hydrogen. The principle of this embodiment of the
invention is that only the coupling reagent is employed
in the cycle, and the extent of reaction is limited for
5   example by limiting reaction times so that all of the
original formed polypeptide does not react. As a
result, after the cycle has been moved to the acid step,
the reaction mixture produced will contain:

 1. Unreacted PITC,

10 2. The reaction product of PITC and the terminal
amino acid residue with which it has reacted (PTC-
polypeptide),

 3. Unreacted original formed polypeptide,

 4. A polypeptide with one less amino acid residue
15 than the original polypeptide.

It will be apparent that by suitable adjustment of
reaction conditions, continued repetition of the cycle
any selected number of times will produce a desired
peptide ladder similar to the ladder produced in the
20   procedure which employs both coupling and terminating
reagents except that the polypeptide members of the
ladder are not end blocked with a terminating reagent.
This process is similarly applicable to a mixture of
polypeptides.

Another procedure for generating a peptide ladder

with only one reagent involves termination by side

reaction. In one such process, PITC is employed as a

coupling reagent; and, under controlled conditions of

oxidation, a small amount of PITC terminated polypeptide

is converted to stable PIC terminated peptide to form a

peptide ladder after a selected number of cycles. The

key to this aspect of the invention is the controlled

oxidation of a small amount of the PITC terminated

polypeptide to form PIC terminated polypeptide which is

stable, or essentially stable, under subsequent

reactions conditions.

To describe the process with more specificity, the

reaction steps are as follows:

    1. React the polypeptide to be sequenced

    under basic conditions with an excess of PITC

    to convert substantially all of the

    polypeptide to PITC terminated polypeptide

    (PTC-polypeptide).

    2. React the PTC-polypeptide with a

    controlled amount of oxygen to convert a

    small portion of the PTC-polypeptide, say 5%,

    to PC-polypeptide while leaving the balance

    unchanged.

3.   Cycle the mixture to the acid step to
cleave the PITC bound terminal amino acid
from the PTC-polypeptide and leave a
polypeptide with one less amino acid residue
than the original polypeptide.

4.   Repeat the cycle any selected number of
times to generate a peptide ladder for mass
spectrometric analysis.

A very significant practical advantage of the
process of this invention is that it is possible to
sequence a plurality of peptides in one reaction system.
This advantage arises principally from the high degree
of accuracy that is possible because of the recent
advances in mass spectroscopy.

This aspect of the invention will be understood by
reference to Figs. 12A and 12B which show a suitable
device for producing a plurality of peptide ladders. In
the figure, 1 is a reaction support member shown in the
form of a cylinder with a holding basin 2 and a through
bore 3 permitting the passage of chemicals. A series of
absorbent members or discs 4, for example absorbent

membranes are supported by a thin filter member 5 which

may be simply a glass fiber or other suitable filter

material.

        In practice, the support member would be in a

5   closed system adapted to permit the appropriate

        reactants for the preparation of a peptide ladder on

        each disc to contact each polypeptide to be sequenced.

        After each step of the cycle, the reactants exit the

        support member through the bore 3. The reactants are

10   delivered to the reaction zone by any conventional

        pumping system of the type employed to collect reactants

        from a series of reservoirs, mix them and pass the

        mixture through a delivery nozzle.

        Sequencing of formed polypeptides on samples

15   immobilized on a solid support, as in the this

        embodiment of the invention is especially advantageous

        because it is applicable to very small amounts of total

        sample and because there are reduced handling losses and

        increased recoveries.

20   As applied to the system illustrated in the

        figures, any convenient number of polypeptides to be

        sequenced are separately absorbed on separate discs 4

        which may be, for example, an absorbent membrane such as

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the cationic, hydrophilic, charge modified

polyvinylidene fluoride membrane available from

Millipore Corp. as Imobilon CD.

5   The discs are spaced apart on the filter paper 5

which is supported over the through bore 3 on support

member 1 which is then placed in a closed system to

conduct the controlled cyclic reactions appropriate to

the production of a peptide ladder in accordance with

this invention.

10   The amount of polypeptide absorbed on each segment

may be as small as one picomole or even less.

Generally, it is from about 1 to about 10 picomoles.

           In a typical operation, 1 to 10 picomoles of each

polypeptide are separately absorbed on the selected

15   membrane discs and placed separately on the filter paper

which is then placed on the support member as shown. The

peptides are subjected to the PITC/PIC/base/acid cycle

described above to generate a peptide ladder on each

disc. Each separate peptide ladder containing mixture

20   to be analyzed may be extracted from each separate

membrane with an organic solvent containing a small

amount of surfactant. One useful extraction solvent is

2.5% trifluoroacetic acid in a 1:1 mixture of

acetonitrile and 1-O-n-octyl-β-glucopyranoside.

Fig. 14 shows the spectrum obtained using the

5   absorbent membrane technology coupled with incomplete

termination described above. To generate the peptide

ladder which was analyzed, 50 picomoles of [Glu-1]

fibrinopeptide B on Immobilon-CD membrane was applied to

ABI-471A protein sequencer (Applied Biosystem). The

10   sequencer was programmed using 5.5 minute cycle time

with a cartridge temperature of 56°C so as to insure

incomplete reaction at each cycle. Six cycles were

performed. Under these conditions, a reaction yield of

about 56% was estimated. The resulting peptide ladder

15   is comprised of free N-terminal amines.

This example illustrates the speed with which the

sequencing can be performed. Similar spectra were

obtained with a total loading of only 1 picomole of

polypeptide on the membrane.

20   Although this multiple, simultaneous, sequence

analysis of separate formed polypeptides utilizing the

same chemical reagents for separate reactions with the

said polypeptides has been specifically described by

reference to the use of a mixture of specific coupling

and terminating reagents in the same reaction zone, it

will be apparent that the process is equally applicable

to the other processes described above.

5   The system is, of course, applicable to the use of

only one disc for the sequencing of a polypeptide or

polypeptide mixture.

Although the discs are shown separately on the

support, they may also be stacked or replaced with a

10   column of suitably absorbent packing materials.

Further, there may be a number of support members

in one device and the chemicals fed to the separate

support members through a manifold system so that

instead of only one reaction zone, there may be a

15   plurality of reaction zones to still further increase

the number of polypeptides which can be simultaneously

sequenced.

An especially important embodiment of this

invention is that it provides a method of locating

20   covalent modifications on a polypeptide chain

particularly post translational modifications of

biologically important products which on chemical or

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enzymatic hydrolysis produce polypeptides which are phosphorylated, aceylated, glycosylated, cross-linked by disulfide bonds or otherwise modified. Such polypeptides are referred to in this specification and claims as "modified polypeptides".

The inability to directly identify, locate, and quantify modified amino acid residues such as phosphorylated residues in a modified polypeptide is a major shortcoming of standard sequencing methods, and has imposed major limitations on currently important areas of biological research, such as mechanisms of signal transduction. The process of this invention has general application to the direct identification of post-translation modifications present in a peptide chain being sequenced. A modified amino acid residue that is stable to the conditions used in generating the peptide ladder from a formed peptide reveals itself as an additional mass difference at the site of the covalent modification. As described above, from the mass difference, both the position in the amino acid sequence and the mass of the modified amino acid can be determined. The data generated can provide unambiguous identification of the chemical nature of the post translational modification.

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A typical example of this aspect of the invention
is the analysis of both phosphorylated and
unphosphorylated forms of the 16 residue peptide
LRRASGLIYNNTLMAR amide prepared by the method of

5   Schnolzer et al (9) containing a phosphorylated serine
residue prepared by enzymatic reaction using 3', 5'-
cyclic AMP-dependent kinase. After ten cycles of
PITC/PIC chemistry on each form of the peptide using the
procedures described above and illustrated in Example 1,

10   the two separate sequence-defining fragment mixtures
(peptide ladders) were each read out by laser desorption
mass spectrometry. The resulting protein ladder data
sets are shown in Figs. 13A and 13B. Again, the mass
differences define the identity and order of the amino

15   acids. For the phosphopeptide (Fig. 13A), a mass
difference of 166.7 daltons was observed for the fifth
amino acid from the N-terminal, compared with the mass
difference of 87.0 for the same residue in the
unphosphorylated peptide (Fig. 13B). This measured mass

20   difference corresponds to a phosphyorylated serine
residue, calculated mass 167.1 daltons. Thus, the
protein ladder sequencing method has directly identified
and located a Ser(Pi) at position five in the peptide.
There was no detectable loss of phosphate from the

phosphoserine residue, which has been regarded in the
art as the most sensitive and unstable of the
phosphorylated amino acids.

Altough only ten cycles of ladder generating
chemistry were performed, sequence-defining fragments
corresponding to eleven residues were observed,
apparently arising from a small amount of premature
cleavage (10). This side reaction which can have
serious consequences for standard Edman methods, has no
effect on the ladder sequencing approach.

A specific and very important advantage of this
invention is that it is not limited to analysis of one
polypeptide. Mixtures of polypeptides can be analyzed
simultaneously in one reaction vessel. Each polypeptide
will give a separate spectrum as shown in idealized form
in Fig. 4. In this figure, the molecular masses of the
original components of the mixture differ by any
arbitrary mass difference. Each of the separate spectra
can be analyzed as described above even though there may
be appreciable overlapping in molecular mass among the
polypeptides to be sequenced. This will be clear from
the figure. As a result, it is possible to sequence

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proteins by analyzing mixtures of polypeptides obtained

by chemical or enzymatic hydrolysis of the protein. The

process can be outlined as follows:

Protein sample in quantities of nanomoles or less

5   enzymatic or
                                               chemical
                                               hydrolysis

fragments

10   separate - e.g.
                                               by HPLC or gel
                                               electrophoresis

collection of separated peptides

                                               parallel cyclic
                                               ladder
15   generating
                                               chemistry

mixture of peptide ladders

                                               mass
                                               spectrometry
20   readout

analysis of data
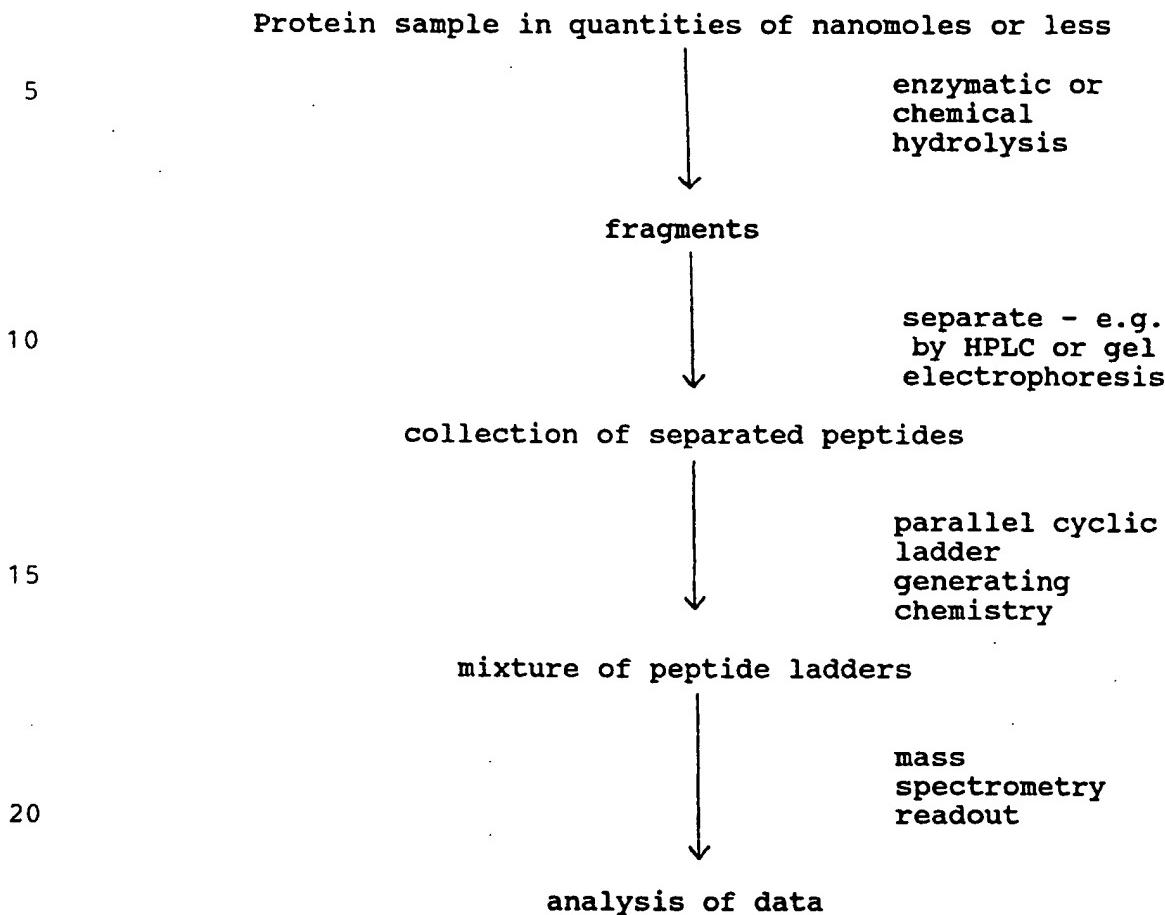
In most cases, gel electrophoresis will be employed

to separate proteins and HPLC to separate polypeptides.

Thus, for example, a protein mixture can be separated

25   into its protein components by electrophoresis and each

separate component sequenced by digestion into

polypeptides, separation and ladder sequencing in

accordance with the process of this invention to yield

data from which the sequence of the entire protein can

be deduced. The process of the invention may also be

employed to obtain extensive data relating to the

5   primary structure of intact proteins at their amino or

carboxy terminals.

There follows a description of the application of

this invention to a forming peptide.

Stepwise solid phase peptide synthesis involves the

10   assembly of a protected peptide chain by repetition of a

series of chemical steps (the "synthetic cycle") which

results in the addition of one amino acid residue to an

amino acid or peptide chain bound to a support, usually

a rsin such as methylbenzhydrylamine. The final

15   polypeptide chain is built up one residue at a time,

usually from the C-terminal, by repetition of the

synthetic cycle. As is well known to peptide chemists,

the solid phase synthetic method does not always proceed

according to plan. For any of a number of reasons, some

20   of the polypeptide formed may terminate before the final

product is produced. For example, a synthesis designed

to produce a polypeptide containing twenty amino acid

residues may produce as side products a variety of

polypeptides containing lesser numbers of amino acid

residues, e.g. tripeptides, octapeptides and

dodecapeptides.

To utilize the advantages of this invention in

5   solid phase synthesis, polypeptide-resin samples are

collected after each cycle of amino acid addition.

Mixing approximately equal amounts of all samples

obtained in the course of a synthesis yields a peptide

ladder containing all possible lengths of resin bound

10   polypeptide. Cleavage of the resin from such a mixture

produces a mixture of free polypeptide chains of all

possible lengths containing a common carboxy or amino

terminal. Usually, stepwise solid phase synthesis

proceeds starting from the carboxy terminal. In these

15   cases, the resulting peptide ladder will contain

polypeptides all having a common carboxy terminal.

Consideration of the steps involved in the

production of a heptapeptide will explain the procedure.

If the heptapeptide to be produced is of the structure:

20   Ala^1-Val-Gly-Leu-Phe-Ala-Gly^7,

the first synthetic step is the attachment of Gly to the

resin, usually with a spacer molecule between the resin

and the Gly. The next step is the attachment of N^{α} -

blocked Ala to the Gly following well known, coupling

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and deblocking procedures so that the synthesis is

controlled. The cycle is repeated to form the

heptapeptide on the resin from which it may be isolated

by standard methods.

 5   In accordance with the procedure of this invention,

a small sample of polypeptide attached to resin is

removed after each cycle. After completion of the

synthesis, the seven samples are added together to

produce a peptide ladder which contains the following

10   components.

                        Gly-Resin

                     Ala-Gly-Resin

                   Phe-Ala-Gly-Resin

                 Leu-Phe-Ala-Gly-Resin

15   Gly-Leu-Phe-Ala-Gly-Resin

               Val-Gly-Leu-Phe-Ala-Gly-Resin

             Ala-Val-Gly-Leu-Phe-Ala-Gly-Resin

        The mixture is then treated, for example with

hydrogen fluoride to generate a resin-free peptide

20   ladder which is analyzed mass spectrometrically to

assure that the final heptapeptide is of the desired

amino acid structure.

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One possible type of side reaction in stepwise solid phase synthesis is low level blocking at a particular residue (step) in the synthesis.

It will be apparent that each has occurred and mixed separate sample collected subsequent to the step at which a side reaction such as low level blocking has occurred above during the assembly of the final polypeptide will contain a portion of such terminated side product with the result that the amount of such terminated peptide is amplified in the final mixture as prepared for mass spectrometric analysis. Thus, for example, if for some reason such as low level blocking there was a termination of some polypeptide at the decapeptide stage in a synthesis designed to produce a 20-residue polypeptide, the sample from each subsequent synthetic cycle would contain terminated decapeptide and the final analytical sample would contain a 10-fold amplification of this side product. The information obtained by this method of analysis is very useful in designing optimum procedures for synthesizing polypeptides, especially those of high molecular weight. One adaptation of this invention to solid phase synthesis is illustrated in Example 2.

Optionally, the peptide resin samples collected as described above may be assayed colorimetrically, for example by a ninhydrin procedure to determine reaction yields prior to mixing to form a peptide ladder. This

5   procedure provides a complimentary method of controlling and assessing the process.

In the foregoing process, a sample of polypeptide attached to the resin is collected at each step of the synthetic cycle for the preparation of the final

10   analytical mixture. An alternative procedure for preparing the final sample is deliberate termination of a small portion of the forming peptide at each step of the synthetic cycle followed by removal of all of the peptides from the resin to form the analytical mixture

15   directly.

This can be accomplished by utilizing, instead of one reversibly blocked amino acid residue at each step in the cycle, a mixture of the selected amino acid residue one portion of which is stable under the

20   reaction conditions, another portion of which is susceptible to removal of the blocking group under controlled conditions.

If, for example, the amino acid residue to be added
to the forming polypeptide is alanine, the peptide bond
could be formed utilizing a mixture of Boc-alanine and
Fmoc-alanine in which the carboxyl group is in the

5   appropriate form for reaction, for example in the form
of an hydroxybenzotriazole ester. After the peptide
bond has been formed, one of the blocking groups, the
removable group, can be removed under conditions such
that the other blocking group remains intact.

10   Repetition of this cycle will result in the formation of
the desired polypeptide on the resin together with a
peptide ladder comprising a series of polypeptides each
member of which is joined to the resin and is terminated
by the selected blocking group.

15   The procedure will be more readily understood by
reference to the preparation of a specific polypeptide
such as:

$$Gly^1\text{-Phe-Ala-Leu-Ile}^5.$$

The chemistry involved in the preparation of such

20   pentapeptide is standard solid phase polypeptide
synthesis applied in such a manner as to produce a
peptide ladder. As applied to this invention, by way of
example, the C-terminal amino acid residue would be

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joined to the resin, typically through a linker, as a
mixture containing a major proportion of t-Boc-
isoleucine and a minor proportion of Fmoc-isoleucine,
e.g. in a 19:1 ratio.

5   The t-Boc blocking group is next removed with an
acid such as trifluoroacetic acid. Since the Fmoc group
is stable under acid conditions the Fmoc-isoleucine
attached to the resin will retain its blocking group and
will be stable to all subsequent reactions.

10   In the next step of this synthesis, a 19:1 mixture
of Boc- leucine and Fmoc-leucine will be joined to the
Ile-Resin, and the Boc blocking group selectively
removed under acid conditions. As a result of this step
in the synthetic cycle, the state of the resin may be
15   indicated by:

                    Fmoc-Ile-Resin

                 Fmoc-Leu-Ile-Resin

                  Leu-Ile-Resin

          Repetition of these reactions will result in a
20   final resin mixture comprising a peptide ladder which
may be represented by:

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<div align="center">

Fmoc-Ile-Resin

Fmoc-Leu-Ile-Resin

Fmoc-Ala-Leu-Ile-Resin

Fmoc-Phe-Ala-Leu-Ile-Resin

Fmoc-Gly-Phe-Ala-Leu-Ile-Resin

Gly-Phe-Ala-Leu-Ile-Resin

</div>

This peptide mixture is removed from the resin by standard solid phase procedures which, optionally, will also remove the Fmoc group to produce an analytical sample ready for analysis by mass spectroscopy as described above.

The peptide ladder can also be formed by the reverse procedure of employing Fmoc as the removable group and t-Boc as the terminating group.

The adaptation of this invention to solid phase synthesis techniques is illustrated in Example 3 and Fig. 11

Any blocking group stable to the conditions of chain assembly synthesis can be used in this application of the invention. For example, acetic acid could be added to each reversibly N-protected amino acid in a

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stepwise solid phase synthesis in an amount suitable to

cause a few percent permanent blocking of the growing

peptide chain at each step of the synthesis. The mass

of the blocking group is without effect on the ability

5   to read out the sequence of the peptide synthesized

since the readout relies on mass differences between

adjacent members of the polypeptide series as described

above.

Using the procedures described, each individual

10   resin bead carries the mixture of target full-length

peptide and the peptide ladder. Typically each bead

carries from 1 to 10 or more picomoles of polypeptides.

Thus, cleavage of the products from a single bead

permits the direct determination of the sequence of the

15   polypeptide on that bead.

It is recognized that the foregoing procedures are

described in an idealized form which does not include

possible interference by other functional groups such as

the hydroxyl group in tyrosine and serine, the "extra"

20   carboxyl groups in dicarboxylic amino acids or the

"extra" amino groups in dibasic amino acids. This

method of description has been adopted to avoid

unnecessarily lengthening the specification. The

artisan will recognize the problems which will be

introduced by the other functional groups and will know
how to deal with them utilizing techniques well known to
peptide chemists.

5   It will also be recognized that the procedures
described have been applied to relatively small
polypeptides. They are equally applicable to large
polypeptides. For example, if the forming polypeptide
is one which contains twenty or more amino acid
residues, it may be expedient to sequence the
10   pentapeptide, the decapeptide and the pentadecapeptide
to be certain that the synthesis is going according to
plan.

       A variety of other chemical reaction systems can be
employed to generate peptide ladders for analysis in
15   accordance with this invention.

       It will be recognized that there are a number of
significant advantages to the processes of this
invention. For example, the demands on yield of the
chemical degradation reactions are much less stringent
20   and more readily achieved than by wet chemical stepwise
degradation techniques such as the Edman degradation in
which low molecular weight derivatives are recovered and
analyzed at each chemical step. Other advantages

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include accuracy, speed, convenience, sample recovery,

and the ability to recognize modifications in the

peptide such as phosphorylation. Relatively

unsophisticated and inexpensive mass spectrometric

5   equipment, e.g. time of flight; single quadrupole; etc.

can be used.

By employing the process of this invention, it is

routinely possible to sequence polypeptides containing

10 or more amino acid residues from one picomole, or

10   even a smaller amount of a polypeptide in one hour or

less including cyclic degradation, mass spectrometry,

and interpretation.

The processes described may be readily automated

i.e., carried out for example in microtiter plates,

15   using an x, y, z chemical robot. Furthermore, the

determination of amino acid sequence from mass

spectrometric data obtained from the protein sequencing

ladders is readily carried out by simple computer

algorithms. The process of the invention therefore

20   includes computer read-out of the spectra of the peptide

ladders produced.

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The skilled artisan will recognize that there are
some limitations to the process of the invention as
described above.

For example, some pairs of amino acids such as
leucine and isoleucine have the same molecular weights.
Therefore, they can not be distinguished by mass
differences of terminated polypeptides in a series.
There are several procedures for avoiding this
difficulty. One is to differentiate them by CDNA
sequencing. They are highly degenerate codons, so they
can be accommodated by inosine substitution in DNA
probes/primers for isolation/identification of the
corresponding gene. This limitation will have little
impact on practical application of the invention.

Further, several amino acids differ by only 1 amu.
This places stringent requirements on accuracy of mass
determination. However, this invention utilizes a
determination of mass differences between adjacent
peaks, not a determination of absolute masses. Since
mass differences can be determined with great accuracy
by mass spectroscopy, the limitation will also be of
little practical significance.

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Finally, samples which are blocked at the amino or carboxy terminal may not be susceptible to the generation of peptide ladders. This problem can be circumvented by chemical or enzymatic fragmentation of

5   the blocked polypeptide chain to yield unblocked segments which can be separately analyzed.

The following non-limiting examples are given by way of illustration only and are not to be considered as limitations of the invention many apparent variations of

10   which may be made without departing from the spirit or scope thereof.

Example 1

Sequencing of [Glu[1]]Fibrinopeptide B

[Glu[1]]Fibrinopeptide B was purchased from Sigma

15   Chemical Co. (St. Louis, Mo.). The reported sequence was: Glu[1]-Gly-Val-Asn-Asp[5]-Asn-Glu-Glu-Gly-Phe[10]-Phe-Ser-Ala-Arg[14]. Matrix assisted laser desorption mass spectrometry gave MW 1570.6 dalton (Calculated: 1570.8 dalton) and showed high purity of the starting peptide.

20   A mixture of PITC plus 5% v/v phenylisocyanate PIC was used in the coupling step. PIC reacts with the NH_2- of a polypeptide chain to yield an N^α-phenylcarbamyl-peptide which is stable to the conditions of the Edman

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degradation. A modification of a standard manual Edman

degradation procedure (6) was used. All reactions were

carried out in the same 0.5mL polypropylene microfuge

tube under a blanket of dry nitrogen. Peptide

(200pmoles to 10 nmole) was dissolved in 20ul of

pyridine/water (1:1v/v; pH10.1); 20uL of coupling

reagent containing

PITC:PIC:pyridine:hexafluoroisopropanol (20:1:76:4 v/v)

was added to the reaction vial. The coupling reaction

was allowed to proceed at 50°C for 3 minutes. The

coupling reagents and non-peptide coproducts were

extracted by addition of 300uL of heptane:ethyl acetate

(10:1v/v), gentle vortexing, followed by centrifugation

to separate the phases. The upper phase was aspirated

and discarded. This washing procedure was repeated

once, followed by washing twice with heptane:ethyl

acetate (2:1v/v). The remaining solution containing the

peptide products was dried on a vacuum centrifuge. The

cleavage step was carried out by addition of 20uL of

anhydrous trifluoroacetic acid to the dry residue in the

reaction vial and reaction at 50°C for 2 minutes,

followed by drying on a vacuum centrifuge. Coupling-

wash-cleavage steps were repeated for a predetermined

number of cycles. The low MW ATZ/PTH derivatives

released at each cycle were not separated/analyzed.

Finally, the total product mixture was subjected to an

additional treatment with PIC to convert any remaining

unblocked peptides to their phenylcarbamyl derivatives.

In this final step, the sample was dissolved in 20uL of

trimethylamine/water (25%wt/wt) in pyridine (1:1v/v);

5   20uL of PIC/pyridine/HFIP (1:76:4v/v) was added to the

reaction vial. The coupling reaction was carried out at

50°C for 5 min. The reagents were extracted as

described above. After the last cycle of ladder

generating chemistry, the product mixture was dissolved

10   in 0.1% aqueous trifluoroacetic acid: acetonitrile (2:1,

v/v). A 1uL aliquot (250pmol total peptide, assuming

no losses) was mixed with 9uL of $\propto$-cyano-4-hydroxy-

cinnammic acid (5g/L in 0.1% trifluoroacetic acid:

acetonitrile, 2:1 v/v), and 1.0uL of this mixture of

15   total peptide products (25pmol) and matrix was applied

to the probe tip and dried in a stream of air at room

temperature. Mass spectra were acquired in positive ion

mode using a laser desorption time-of-flight instrument

constructed at The Rockefeller University (7). The

20   spectra resulting from 200 pulses at a wavelength of

355nm, 15 mJ per pulse, were acquired over 80 seconds

and added to give a mass spectrum of the protein

sequencing ladder shown in Fig. 7. Masses were

calculated using matrix peaks of known mass as

25   calibrants.

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<u>Peptide sequence read-out</u>. Positive ion (MALDMS)

spectra of [Glu[1]]Fibrinopeptide B is shown in Fig. 6. A

protonated molecular ion [M+H] was observed at m/z

1572.5 (calculated value is 1571.8).

5   Its positive ion MALDMS spectrum of the reaction

mixture obtained after seven cycles is shown in Fig. 6.

Each of the peaks in the spectrum represents a related

phenylcarbamoylpeptide derivative in the peptide ladder

(except a few peaks which will discussed later). The

10   amino acid sequence can be easily read-out from the mass

difference of adjacent two peaks. for instance, the

mass difference are 129.1, 56.9, and 99.2 between peaks

at m/z 1690.9 and 1561.8, peaks at m/z 1561.8 and 1504.9

and peaks at m/z 1504.9 and 1405.7. Which correspond to

15   glutamic acid (ca. 129.12), glycine (ca. 57.05) and

valine (ca. 99.13) residues, respectively. One set of

paired peaks gives mass difference 119.0 (1062.1-943.1)

which corresponds to the phenylcarbamoyl group. In

other words, these two peaks represent one piece of

20   peptide with or without phenylcarbamoyl group. Peak at

m/z 1553.8 corresponds partially blocked peptide with

pyroglutamic acid at the N-terminus. This results from

cyclization of the N-terminal Glu under the reaction

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conditions used. Such products are readily identified
from the accurately measured mass and know chemical
reaction tendencies.

Example 2

Stepwise solid phase synthesis of the 99 amino acid
residue polypeptide chain corresponding to the monomer
of the HIV-1 protease (SF2 isolate):

PQITLWQRPLVTIRIGGQLKEALLDTGADDTVLEEMNLPGKWKPKMIGGIGGFIKVR

QYDQIPVEI(Aba)GHKAIGTVLVGPTPVNIIGRNLLTQIG(Aba)TLNF^{99}

[where Aba = α-amino-n-butyric acid] was undertaken.

Highly optimized Boc-chemistry instrument-assisted
stepwise assembly of the protected peptide chain was
carried out on a resin support, according to the method
described by S.B.H. Kent (8). Samples (3-8mg, about
1umole each) were taken after each cycle of amino acid
addition. The protected peptide-resin samples were
mixed in three batches of consecutive samples: (number
corresponds to the amino acid after which sample was
taken, i.e. residue number in the target sequence.) 99-
67; 66-33; 32-1. The first such mixture contained the
peptides:

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                              99-Resin

                            98-99-Resin

                          97-98-99-Resin

                        96-97-98-99-Resin

5   . . . .(etc.). . . .

            70. . . . 96-97-98-99-Resin

         69-70. . . . 96-97-98-99-Resin

      68-69-70. . . . 96-97-98-99-Resin

   67-68-69-70. . . . 96-97-98-99-Resin

10   Similarly for the other two mixtures. The mixed batches

     of peptide-resin were deprotected and cleaved with HF (1

     hours, at 0°C, plus 5% cresol/5%/thiocresol). The

     products were precipitated with diethyl ether, dissolved

     in acetic acid-water 950/50%, v/v) and then lyophilized.

15   Each peptide mixture was dissolved in 0.1% TFA, 1

     uL of the peptide mixture (10 uM per peptdie component)

     was added to 9uL of 4-hydroxy- -cyanocinnamic acid in a

     1:2 (v/v) ratio of 30% acetonitrile/0.1% aqueous

     trifluoroacetic acid. 0.5uL of the resulting mixture

20   was applied to the mass spectrometer probe and inserted

     into the instrument (7). The spectra shown in Figs. 8

     and 9 are the result of adding the data of each of 100

laser shots performed at a rate of 2.5 laser

shots/second.   Figure 8 shows the mass spectrum obtained

from the mixture resulting from cleaving mixed samples

from residues 99-67 of the synthesis.   Fig. 9 shows the

5   mass spectrum obtained from the mixture resulting from

cleaving mixed samples from residues 66-33 of the

synthesis.   Table 1 shows the measured mass differences

between consecutive peaks of a selection of these peaks

and compares them with the mass differences calculated

10   from known sequences of the target peptides.   The

agreements are sufficiently close to allow confirmation

of the correctness of the synthesis.

Figure 11 shows mass spectra of the mixture

obtained from mixed samples from residues (66-33) of the

15   synthesis.

The sequence of the assembled polypeptide chain can

be read out in a straightforward fashion from the mass

differences between consecutive peaks in the mass

spectra of the peptide mixture.   This confirmed the

20   sequence of amino acids in the peptide chain actually

synthesized.   The identity of the amino acids as

determined by such mass differences is shown in Table 1

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Table 1. The identify of amino acid by the mass differences in protein ladder sequencing
using matrix-assisted laser desorption mass spectrometry.

Amino Acid	Mass Difference (Measured, Da)	Deviation	Amino Acid	Mass Difference (Measured, Da)	Deviation
Leu[33]	113.3	0.1	Asp[60]	114.8	-0.3
Glu[34]	129.7	0.6	Gln[61]	128.7	0.6
Glu[35]	129.5	0.4	Ile[62]	113.2	0.0
Met[36]	130.8	-0.4	Pro[63]	97.0	-0.1
Asn[37]	115.0	0.9	Val[64]	99.4	0.3
Leu[38]	112.4	-0.8	Glu[65]	128.6	-0.5
Pro[39]	97.9	0.8	Ile[66]	113.3	0.1
Gly[40]	56.1	-0.9	Aba[67]	84.9	-0.2
Lys[41]	128.1	0.0	Gly[68]	57.0	0.0
Trp[42]	186.4	0.2	His[69]	137.3	0.2
Lys[43]	128.2	0.0	Lys[70]	127.8	-0.4
Pro[44]	97.1	0.0	Ala[71]	71.4	0.3
Lys[45]	128.0	-0.2	Ile[72]	113.4	0.2
Met[46]	131.9	0.7	Gly[73]	56.8	-0.2
Ile[47]	112.6	-0.6	Thr[74]	101.1	0.0
Gly[48]	57.9	0.9	Val[75]	99.2	0.1
Gly[49]	56.3	-0.7	Leu[76]	113.1	-0.1
Ile[50]	112.4	-0.8	Val[77]	99.1	0.0
Gly[51]	57.6	0.6	Gly[78]	57.1	0.1
Gly[52]	57.5	0.5	Pro[79]	97.2	0.1
Phe[53]	147.3	0.1	Thr[80]	101.1	0.0
Ile[54]	112.5	-0.7	Pro[81]	97.1	0.0
Lys[55]	128.9	0.8	Val[82]	99.2	0.1
Val[56]	99.0	-0.1	Asn[83]	113.8	-0.3
Arg[57]	156.2	0.0	Ile[84]	113.4	0.2
Gln[58]	128.4	0.3	Ile[85]	113.1	0.0
Tyr[59]	162.6	-0.6	Gly[86]	57.1	0.0

In addition, terminated by-products (where the
peptide chain has become blocked and does not grow
anymore) are present in every peptide-resin sample taken
after the step in which the block occurred. Thus, there

5   is an amplification factor equal to the number of resin
samples in the batch after the point of termination.
This can be seen in Fig. 10 (samples #66-33) which
contains a peak at 3339.0. This corresponds to the
peptide 71-99, 3242.9 (N-terminal His71) plus 96.1

10   dalton. The characteristics mass, together with
knowledge of the chemistry used in the synthesis
identifies the blocking group as CF3CO-(97.1-H = 96.1
dalton). The observed by product is the
trifluoroacetyl-peptide, N^{α}-Tfa-(71-99). The ratio of

15   the amount of this component to the average amount of
the other components is about 2:1. There were 34
samples combined in this sample. Thus, the terminated
byproduct N^{α}-Tfa-(71-99) had occurred at a level of
about 5mol%. This side reaction, specific to the N-

20   terminal His-peptide chain, has not previously been
reported. This illustrates the important sensitivity
advantage provided by this amplification effect in
detecting terminated peptides. Such byproducts are not
readily detected by any other means.

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Example 3

Boc/Fmoc Terminations

Synthesis of the peptide LRRAFGLIGNNPLMAR-amide was
performed manually on a 0.2 mmol scale using p-
methylbenzhydrylamine resin and 0.8 mmoles amino acid
(95 mol%
N-α-Boc, 5 mol% N-α-Fmoc) according to the in situ
neutralization methods of Schnolzer et al (9). The
following side chain protecting groups were used: Boc-
Arg, tosyl; Fmoc-Arg, 2,3,6-trimethyl-4-
methoxybenzenesulfonyl (Mtr). Fmoc-Arg(Mtr) was used
for its greater stability in trifluoroacetic acid (TFA).
After completion of the chain assembly, Fmoc groups were
removed using 50% piperidine/DMF, followed by Boc group
removal in TFA. The peptide fragments were then cleaved
from the resin by treatment with HF-10% p-cresol (0°C, 1
hour). The resulting crude peptide products were
precipitated and washed with ether, dissolved in 50%
acetic acid, diluted with water and lyophilized. The
mass spectra of the reaction mixture thus produced is
shown in Fig. 11.

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Example 4

Post-ninhydrin Experiment The machine-assisted

assembly of the peptide LRRASGLIYNNPLMAR-amide was

performed according to the in situ neutralization

5   methods of Schnolzer and Kent (9) on a 0.25 mmol scale

using MBHA resin and 2.2 mmol N-α-Boc amino acids. The

following side chain protecting groups were used: Arg,

tosyl; Asn, xanthyl; Ser, benzyl(Bzl); Tyr,

bromobenzyloxycarbonyl(BrZ). Resin samples were

10   collected at each step in the synthesis and each sample

was individually subjected to the quantitative ninhydrin

reaction. These samples were then pooled and the Boc

groups removed in neat TFA. Cleavage of the peptide

fragments from the resin was performed by treatment with

15   HF-10% p-cresol (OC, 1 hour). The resulting crude

peptide products were precipitated and washed with

ether, dissolved in 50% acetic acid, diluted with water

and lyophillized. The mass spectrum of the mixture is

shown in Fig. 15.

20   CITATIONS

        The following publications are referred to in this

specifications. The complete disclosure of each of them

is hereby incorporated by references.

54

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